

1   **Toward establishing model organisms for marine protists: successful transfection protocols**  
2   **for *Parabodo caudatus* (Kinetoplastida: Excavata)**

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16   Running title: Protist transfection

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20 **Originality-Significance Statement:** This study is the first experimental evidence comparing  
21 three different electroporation methods for transfection of microbial eukaryotes. Our recently  
22 developed microfluidic transfection technology and square wave protocols resulted in higher  
23 efficiency and reproducibility compared to the traditional exponential decay electroporation.  
24 *Parabodo caudatus* has the potential to be a genetically tractable model protist organism because  
25 it grows quickly and relatively easily in the laboratory, and its transparent cell membrane facilitates  
26 observations of measurable phenotypic traits in genetically transformed cells. *P. caudatus* is  
27 abundant in marine and freshwater environments, and is of evolutionary interest for being one of  
28 the free-living close-relatives of parasitic trypanosomes. The development of transfection  
29 protocols for *P. caudatus* will help to further understand evolution, physiology, and ecological  
30 roles of *P. caudatus*.

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32 Key words: Transfection; electroporation; microfluidic; micro-eukaryotes; reporter genes

33 **Summary:**

34 We developed protocols for, and demonstrated successful transfection of, the free-living  
35 kinetoplastid flagellate *Parabodo caudatus* with three plasmids carrying a fluorescence reporter  
36 gene (pEF-GFP with the EF1 alpha promoter, pUB-GFP with Ubiquitin C promoter, and pEYFP-  
37 Mitotrap with CMV promoter). We evaluated three electroporation approaches: 1) a square-wave  
38 electroporator designed for eukaryotes, 2) a novel microfluidic transfection system employing  
39 hydrodynamically-controlled electric field waveforms, and 3) a traditional exponential decay  
40 electroporator. We found the microfluidic device provides a simple and efficient platform to  
41 quickly test a wide range of electric field parameters to find the optimal set of conditions for

42 electroporation of target species. It also allows for processing large sample volumes (> 10 ml)  
43 within minutes, increasing throughput 100 times over cuvettes. Fluorescence signal from the  
44 reporter gene was detected a few hours after transfection and persisted for 3 days in cells  
45 transformed by pEF-GFP and pUB-GFP plasmids and for at least 5 days post-transfection for cells  
46 transformed with pEYFP-Mitotrap. Expression of the reporter genes (GFP and YFP) was also  
47 confirmed using reverse transcription-PCR (RT-PCR). This work opens the door for further efforts  
48 with this taxon and close relatives toward establishing model systems for genome editing.

49

## 50 Introduction

51 Protists are unicellular eukaryotes that are ubiquitous in the marine realm, their molecular  
52 signatures have been described from all marine habitats investigated, and they are recognized as  
53 pivotal members of aquatic microbial communities in models of carbon cycling (Worden et al.,  
54 2015). Phototrophic protists contribute to primary production, and heterotrophic protists shape  
55 organic matter pools and populations of their prokaryotic and eukaryotic prey, thus indirectly  
56 influencing activities at the foundation of microbially-driven major nutrient cycles (Azam and  
57 Malfatti, 2007). Free-living protists exhibit complex interactions with other protists, Metazoa,  
58 Bacteria, Archaea, and viruses. Our understanding of the extent of marine protist diversity has  
59 expanded tremendously over recent decades, due in significant part to information from high  
60 throughput sequencing approaches based on bulk extracted DNA/RNA. Protists also exhibit  
61 symbioses with prokaryotes and with other protists and Metazoa. Marine protist taxa with  
62 sequenced genomes reveal that microeukaryotes can have complex and large genomes that can be  
63 many times greater in size than even the human genome. The function of a significant portion of  
64 those genes remains unknown, and is referred to as “genetic dark matter” (Clark et al., 2013).

65 To uncover the scientific principles that govern the interactions of protists with other  
66 microbes and that mediate nutrient flow in the sea, an understanding of the function of this genetic  
67 “dark matter” facilitated by genetically tractable model representatives is required. Their  
68 development will allow us to systematically decipher the gene–gene and gene–environment  
69 interactions, and to understand processes underlying the roles of certain protists in biogeochemical  
70 cycling and the evolution and ecology of the microbial Eukarya. Low efficiency transfection  
71 protocols exist for a few marine protists; *Ostreococcus tauri*, *Phaeodactylum tricorutum*,  
72 *Amphidinium* sp. and *Symbiodinium microadriaticum* (Te et al., 1998; De Riso et al., 2009; van

Ooijen et al., 2012). Transfection of two dinoflagellates has been successful using silicon carbide whiskers to deliver plasmid constructs (Te et al., 1998), and **transfection of *Perkinsus marinus*, a marine protist parasite, has been achieved using** electroporation (Fernandez-Robledo et al., 2008; Cold et al., 2016). **Recent** gene editing protocols have been established for few diatoms (Hopes et al., 2016; Liu et al., 2016), and progress toward useful forward genetics approaches for choanoflagellates have been established (Hoffmeyer and Burkhardt, 2016). Additional models are needed for more widely distributed, ecologically important free-living lineages. Methods of gene tagging and gene silencing using CRISPR/Cas9 have been developed for a few, mainly parasitic protists (*Trypanosoma cruzi*, *Leishmania* spp., *Plasmodium* spp., *Cryptosporidium parvum*, *Chlamydomonas reinhardtii*) (Lander et al., 2015; Peng et al., 2015; Lander et al., 2016a; Lander et al., 2016b), but again, there is a lack of methods for most protist taxa. Genetic manipulation of marine protists will make it possible to link genes of unknown function to cell behavior (e.g., colony formation, morphogenesis, cell-cell interactions), physiology (e.g., life cycle and reproduction type), particular biogeochemical cycles, and processes of interest, such as, nitrogen and carbon cycling, and production of climate active trace gases or initiation of harmful algal blooms (Gong et al., 2017).

The first step in developing transgenic marine protists is the establishment of reliable and reproducible transfection protocols. Transfection can be accomplished using chemical-based methods (which include lipofection, calcium phosphate, etc.), electroporation, microinjection, biolistic, laserfection/optoinjection, and virus-based methods (Kim and Eberwine, 2010). Electroporation using pulsed electric fields is a technology that has become a powerful and mature tool used for genetic engineering of prokaryotes and eukaryotes. Via electroporation, electrical pulses of a specified voltage and duration transiently disrupt the membrane of cells and deliver

96 foreign DNA (i.e., plasmids) into the cells within milliseconds. With the advent of electroporation  
97 bacterial strains could be transformed with plasmids carrying marker genes (Teissie and Tsong,  
98 1981; Neumann et al., 1982; Josenhans et al., 1998) and methods have evolved since then to  
99 include a wide range of tools including most recently, RNAi and CRISPR/Cas9 (Peng et al., 2015;  
100 Liang et al., 2017). Such approaches can allow researchers to gain an understanding of the role of  
101 a gene of interest in the physiology or behavior of an organism, and in turn, the role the gene plays  
102 in the organism's ecology.

103 While electroporation methods for some bacteria, such as *E. coli*, are technically simple  
104 and thus commonplace, the efficacy of electroporation, broadly speaking, depends on many  
105 variables. The critical parameters in electroporation are the electric field magnitude and pulse  
106 characteristics such as the shape, duration, and number of pulses (Weaver et al., 2012).  
107 Traditionally, electroporation is performed in plastic cuvettes outfitted with parallel plate metal  
108 electrodes. The separation distance between the plate electrodes is fixed at 1 mm, 2 mm, or 4 mm  
109 and the DNA-cell suspension is placed between the electrodes. Upon application of a voltage, an  
110 electric field is generated between the plates at a magnitude equal to the voltage-to-distance ratio  
111 (Corovic et al., 2007). Other experimental parameters affecting the outcome include the DNA  
112 concentration and the electrical conductivity (salt concentration) of the medium surrounding the  
113 cells (i.e., electroporation buffer). Buffers with lower ionic concentrations reduce the arcing  
114 potential (electrical charges that occur due to high or excess salt concentration), reduce deleterious  
115 heating, and generally increase the transfection efficiency (Kotnik et al., 2015). Notably,  
116 eukaryotic cells (including many protists) are highly sensitive to the experimental conditions that  
117 are optimal for electroporation, such as lower ionic concentrations. The low efficiency of

118 electroporation resulting from the myriad of technical challenges has hindered progress in  
119 developing electroporation-based methods appropriate for aquatic protists.

120         Accordingly, we aimed to develop an efficient method for electroporation of marine  
121 protists. For this effort, we selected *Parabodo caudatus*, a free-living biflagellate kinetoplastid  
122 (Parabodonida, Kinetoplastea, Euglenozoa), and free-living close relative of parasitic  
123 trypanosomatid flagellates. *P. caudatus* feeds on bacteria (e.g., *Klebsiella pneumoniae*,  
124 *Escherichia coli*, *Enterobacter aerogenes*, and others). *P. caudatus* cells divide by longitudinal  
125 binary fission. Members of this family have fast growth rates and are easy to culture and maintain  
126 in laboratory. They prey on bacterial cells and are ubiquitous in many environments including  
127 fresh and marine water columns and sediments, seawater from deep-sea hydrothermal vents, and  
128 as contaminants in food (von der Heyden et al., 2004; Tikhonenkov et al., 2016). The genome of  
129 a congener of *P. caudatus*, *Bodo saltans*, revealed that 60% of all coding genes had homologs in  
130 trypanosomatids, evolutionary close relatives to kinetoplastids (Jackson et al., 2008; Jackson et al.,  
131 2016; Oppendoes et al., 2016). The remainder of genes was found to be homologs of genes in other  
132 eukaryotes (i.e., fungi, animals, and plants) but not trypanosomatids, or *Bodo*-specific genes with  
133 no matches to sequences in public databases. These *Bodo*-specific genes were predicted as  
134 hypothetical proteins expressed on the cell surface (Jackson et al., 2008; Jackson et al., 2016;  
135 Oppendoes et al., 2016). These findings suggest that *Parabodo* and other free-living bodonids  
136 represent appealing model organisms for exploring potential roles of those unidentified genes.

137         To determine the efficacy of electroporation as a means of transforming marine protists,  
138 we tested three different transfection technologies. First, we evaluated a commercially available  
139 square-wave technology that has been successfully used to increase transfection rates by 2-3 times  
140 in living cells ranging from very fragile mammalian stem cells (Kaneko et al., 2014) to intact

141 vertebrate embryos (when compared to traditional exponential decay electroporation; (Sanders et  
142 al., 2013)). Very few studies to date, however, have examined electroporation responses of free-  
143 living microeukaryotes, which are markedly different than mammalian cell lines in their robustness  
144 and their transfection amenability (Miyahara et al., 2013). While the exponential decay  
145 electroporation methods enable voltage, resistance, and capacitance to be independently selected,  
146 the square-wave generator enables additional parameters such as pulse duration, pulse number,  
147 and polarity. The square-wave system may be used to apply two types of multi-pulse  
148 electroporation protocols (Fig. 1b). The first sequence uses poring pulses, which are multiple high-  
149 voltage, short duration (5 ms) pulses responsible for formation of the initial pores (membrane  
150 defects) in the cell membrane. The second sequence uses transfer pulses, which are multiple low-  
151 voltage, long duration (50 ms) pulses that deliver the target molecules into cells with minimal  
152 damage. The low-voltage pulses are similar to those used in electrophoresis, which facilitates the  
153 movement of charged molecules such as DNA into the cells.

154       The second method that we evaluated utilizes a novel microfluidic platform for identifying  
155 critical electroporation conditions for successful transformation, recently developed by a subset of  
156 the authors (Garcia et al., 2016). We further developed our microfluidic platform to conduct  
157 continuous flow transformation of microorganisms (Garcia et al., 2017). This technology uses  
158 microfluidic channels with geometric constrictions (see Experimental Procedures section for  
159 physical dimensions) in order to amplify the electric field to achieve electroporation (Fig. 1a). In  
160 our bilaterally converging microfluidic system, a single applied voltage results in a linear electric  
161 field gradient along the length of the microchannel constriction and results in a maximum ~6x  
162 amplification of the applied voltage (1 V = 6 V/cm). Thus, depending on the applied voltage and  
163 the location within the constriction, the strength of the electric field will be able to induce cell



164 electroporation. Square wave pulses are delivered from electrodes with alternating polarity  
165 between the pulses to reduce electrolytic effects at the electrode-buffer interface (Fig. S1). For  
166 example, square wave pulses with 5 ms ON and 5 ms OFF cycles are applied to the microchannel  
167 through the dispensing needle, which results in 50 % of the cells experiencing the electric field  
168 during their transit through the channel. Increasing the duty cycle would result in a larger fraction  
169 of the cells being exposed to the electric field, but also more sample heating. In order to mitigate  
170 potential deleterious heating, the flow rate must be selected carefully in order to remove the heated  
171 sample from the constriction without compromising cell viability. The microfluidic device  
172 conveniently provides a simple platform to efficiently test transfection conditions and optimize  
173 parameters for genetic manipulation of recalcitrant organisms such as many protists.

174       The third system we investigated was a MicroPulser Bio-Rad (CN 165-2100) exponential  
175 decay system (Fig. 1). Among electroporation technologies, exponential decay technologies have  
176 been routinely used in laboratories the longest. Here we report successful transient transfection of  
177 the free-living kinetoplastid flagellate *Parabodo caudatus* with three plasmids carrying fluorescent  
178 protein (FP) reporter genes, using three electroporation approaches: 1) a new microfluidic  
179 transfection system using hydrodynamically-controlled waveforms, 2) a square-wave transfection  
180 system, and 3) traditional exponential decay electroporation. This study is the first experimental  
181 comparison of successful transient transfection of marine microeukaryotes employing three  
182 different electroporation methods. It lays the groundwork for future efforts aimed at stable  
183 transfection with a variety of gene targets, and genetic manipulation of this taxon and its close  
184 relatives.

## 185 **Results**

## 186 **Cell Viability After Cytomix Buffer Incubation**

187 Tolerance of *P. caudatus* to different electroporation buffers was tested. *P. caudatus* cells  
188 were viable after incubation in 50 % and 10 % cytomix (Knight and Scrutton, 1986) buffer  
189 concentrations for at least 15 minutes. However, *P. caudatus* incubated in 100 % cytomix were  
190 viable for about 10 minutes, after which increasing cell mortality was observed. Therefore, all our  
191 subsequent experiments were performed in 50 % cytomix buffer, 10% or 1% seawater, or MilliQ  
192 water in order to maintain sufficient cell viability.

## 193 **Electroporation Buffer Conductivity**

194 The exponential decay (MicroPulser Bio-Rad CN 165-2100) system resulted in arcing at  
195 the highest voltage tested (1000 V), because all cytomix buffer concentrations tested had relatively  
196 high electrical conductivity. However, when the voltage was reduced (800 V, 500 V, and 300 V)  
197 arcing was eliminated and the resulting pulses lasted between 0.7 ms and 1.2 ms. Buffers  
198 composed of 10 % seawater and 1 % seawater both resulted in pulse durations of 0.7 ms and 3.5 ms  
199 at all applied voltages between 1000 V and 300 V. These pulse durations are all shorter than the  
200 typical 5.0 ms that results when using low conductivity buffer, such as MilliQ water at all voltages  
201 tested.

202 For the square wave electroporation system (NEPA21 transfection system, Bulldog Bio),  
203 cytomix buffers at high or low concentrations and the 10 % seawater were too conductive,  
204 resulting in arcing at 150 V and 300 V during a continuous 5.0 ms square pulse (Miyahara et al.,  
205 2013). In contrast, when voltage strength of both poring and transfer pulses was reduced to 99 V,  
206 with multiple 5.0 ms square pulses, the treatment was successful in the 100 % cytomix, 50 %  
207 cytomix, 10 % cytomix, and 10 % seawater buffers (Table 2). Buffers with low conductivity, such

208 as, 1 % seawater and MilliQ water were also able to complete the entire treatment without arcing  
209 at any of the tested voltages (99 V, 150 V, and 300 V).

#### 210 **Post-Electroporation Cell Viability Quantification**

211 Using the exponential decay system, *P. caudatus* cells did not survive exposure to 1000 V  
212 in any of the above mentioned electroporation buffers (Table S1). In contrast, 40-50 % of cells  
213 were viable post-electroporation when a single exponentially decaying pulse was applied at 800 V  
214 ( $E = 4,000$  V/cm) in all tested electroporation buffers. When the maximum voltage was limited to  
215 500 V ( $E = 2,500$  V/cm) cell viability increased to between 60-70 %. Applied voltages of 300 V  
216 ( $E = 1,500$  V/cm) resulted in the highest cell viability of about 80-90 %.

217 Using the square-wave system, we initially tested the same parameters that were  
218 successfully applied for transformation of diatoms (Miyahara et al., 2013). Electroporated cells  
219 exposed to 150 V ( $E = 750$  V/cm) or 300 V ( $E = 1,500$  V/cm) in MilliQ water or 1% seawater  
220 survived. However, these electric fields failed to successfully transform *P. caudatus* with plasmid  
221 DNA, potentially because the transfer pulses used very low voltage (8 V). We therefore tested  
222 several other poring and transfer pulse voltage combinations, pulse numbers, and durations. Cells  
223 of *P. caudatus* electroporated with a maximum applied voltage of 99 V for poring and transfer  
224 pulses ( $E = 500$  V/cm) in any of the investigated electroporation buffers (cytomix, seawater, and  
225 MilliQ water) were viable with no observed cell damage or loss. These parameters were also  
226 successful for establishing plasmid DNA transfection for *P. caudatus*.

227 We did not specifically assess cell viability after applying the microfluidic platform since  
228 cells are exposed to different electric fields with a single applied voltage (Garcia et al., 2016).  
229 Additionally, depending on the duty cycle selected, some of the cells flowed through the device

230 without being exposed to any electric field. Therefore, we tested electric field parameters that  
231 resulted in high cell viability using the exponential decay platform in subsequent experiments in  
232 the microfluidic device.

### 233 **Real-Time Permeabilization Confirmation with SYTOX® Post-Electroporation**

234 We aimed to establish the first transfection protocols for marine protists using the  
235 microfluidic system, and to identify the critical electric field that is required for the onset of  
236 electroporation. Initially, the ability to permeabilize *P. caudatus* cells was tested using the  
237 intercalating dye SYTOX® blue nucleic acid stain, which fluoresces upon binding to intracellular  
238 DNA. We delivered a single pulse with applied voltages of 500 V ( $E_{max} = 3,000$  V/cm) and  
239 1,000 V ( $E_{max} = 6,000$  V/cm) in the absence of flow to expose cells to a narrow range of electric  
240 fields. The fluorescence images depicted in Fig. 2e and 2f confirm the ability to electroporate the  
241 cells at electric fields ranging from 1,000 V/cm – 6,000 V/cm with a pulse duration of about 5 ms.  
242 The extremely high electroporation efficiency at the ideal electric field can be seen in Fig. 2f, in  
243 which the majority of cells in the microfluidic channel have been successfully permeabilized  
244 (Supplementary Video 1). However, because the dead cells are also labelled with SYTOX®, this  
245 electroporation assay does not inform on the upper limit of the electric field within the range where  
246 cells are labelled and still viable. Transfection with plasmid DNA using the microfluidic  
247 technology provided more conclusive evidence of transfection success.

### 248 **Transformation of *P. caudatus* with Plasmids**

249 Circular DNA plasmids pEF-GFP, pUB-GFP, and pEYFP-Mitotrap were introduced  
250 separately into *P. caudatus* using the three different electroporation systems with parameters  
251 presented in Tables 1 and 2. All of our plasmids were expressed in the cytoplasm of *P. caudatus*

252 cells, after they were transcribed in the host's nucleus. In all cases of successful transfection,  
253 transformants were viable and their growth rate was similar to that of wild-type cells. No  
254 morphological differences in the cell shape were detected between the transformed and wild type  
255 cells (Figs. 3 and 4). Expression of the GFP gene, driven by either the EF1 alpha promoter or the  
256 ubiquitin C promoter, and the YFP gene, driven by the CMV promoter, was documented using a  
257 fluorescence microscope 12 hours post-electroporation (Figs. 3 and 4). Expressed GFP signal  
258 levels decreased gradually over the 48 h post-electroporation, but YFP expression was maintained  
259 for 5 days (the longest time that expression was monitored). Microscopy revealed that GFP  
260 expression driven by the ubiquitin C promoter was stronger than the GFP expression pattern driven  
261 by the EF1 alpha promoter. Reverse transcription-PCR performed using RNA isolated from *P.*  
262 *caudatus* cells transfected using the microfluidic system and the pUB-GFP plasmid revealed the  
263 presence of GFP transcripts 3 days post-transfection (Fig. 5). These results clearly indicate that the  
264 pUB-GFP plasmid was delivered into *P. caudatus* nucleus by electroporation and was transcribed  
265 to GFP mRNA *in vivo*.

266 The fluorescence signal resulting from transfection of *P. caudatus* with the pEYFP-  
267 Mitotrap plasmid was stable for 5 days post-transfection. Transcription of the YFP gene was  
268 confirmed by RT-PCR using RNA isolated 5 days post-transfection (Fig. 5). Stability was not  
269 monitored past 5 days in this study. Given optimization of antibiotic selection markers was outside  
270 the scope of this short-term project, post-transfection cultures were maintained in the absence of a  
271 selection marker, and hence were not suitable for long-term observation or experiments to confirm  
272 stable transfection.

273 The microfluidic technique was implemented in the transformation of *P. caudatus*, but  
274 unlike the square-wave and exponential decay systems, this system does not employ a uniform

275 electric field. As cells flow through the microfluidic device, they are exposed to multiple electric  
276 fields, making it challenging to assess viability as a function of a specific, uniform electric field.  
277 Based on our electroporation assays, initial unsuccessful attempts were made using maximum  
278 electric fields of 6,000 V/cm or 9,000 V/cm at the constriction with a 20 % duty cycle (Table S2).  
279 In order to improve the probability of transfection, the duty cycle was increased to 50 % with  
280 maximum electric fields of 750 V/cm, 1,500 V/cm, or 2,250 V/cm. These experiments conducted  
281 with a 50 % duty cycle resulted in transfection efficiencies ranging between 20-30 %. Finally, we  
282 increased the duty cycle to 95 % to increase the fraction of treated cells with maximum electric  
283 fields ranging between 500 V/cm and 3,000 V/cm. Transfection efficiencies ranging between 30-  
284 50 % were also achieved with maximum electric fields of 1,500 V/cm and 2,250 V/cm using 5 ms  
285 pulses in MilliQ water. Additionally, we achieved transfection efficiencies ranging between 20-  
286 30 % using a maximum electric field of 1,000 V/cm with 20 ms pulses in 50 % cytomix buffer in  
287 a straight channel.

288 Transformation efficiencies (percentage of successfully transformed cells) were  
289 comparable for the microfluidic platform and the commercially available square-wave technology.  
290 The microfluidic platform was the most efficient method with 30-50 % of the cells successfully  
291 transformed (Table 1). The square-wave platform resulted in transformation efficiency of  $\geq 40$  %  
292 (Table 2). Finally, the exponential decay electroporation resulted in  $\leq 5\%$  transformation  
293 efficiency making it the least optimal transfection platform evaluated (Table 1).

294

## 295 Discussion

296 In this study we achieved successful transfection of *P. caudatus* using three electroporation  
297 systems; our microfluidic platform, a square-wave system, and traditional exponential decay

298 methods. The process of developing transient transfection protocols in *P. caudatus* involved initial  
299 testing and determination of proper electroporation buffers and parameters (voltage strength, pulse  
300 duration and number). Our results suggest that the type of electroporation buffer is critical for  
301 maintaining cell viability throughout the experiment and is essential for determining the optimum  
302 electric field range. We have demonstrated that successful electroporation conditions were  
303 different for the three electroporation systems utilized, and are largely dependent on the electric  
304 field strength, as well as the number and duration of pulses. Transient transfection was carried out  
305 using three plasmids, pUB-GFP, pEF-GFP, and pEYFP-Mitotrap, which utilize promoters that are  
306 recognizable to most eukaryotes, to determine and optimize electroporation parameters. We now  
307 know that all three promoters, CMV, ubiquitin C, and EF1 alpha, work successfully with *P.*  
308 *caudatus*. Our transient transfection experiments demonstrated the feasibility of introduction and  
309 expression of foreign DNA into *P. caudatus* using each of the three systems (microfluidic, square  
310 wave, and the exponential decay), and the optimal electroporation parameters to apply for future  
311 stable transfection of *P. caudatus*.

312 Although the pEYFP-Mitotrap plasmid includes neomycin resistance as a selection marker  
313 gene and Tom70p as a target gene for the mitochondrial outer membrane (Robinson et al., 2010),  
314 the long-term stability of transfection and the efficacy of transformant selection based on antibiotic  
315 resistance were not examined in our study. Assessing longer-term stability of transfection using  
316 selective marker genes requires initial screening with various antibiotics to determine the most  
317 effective antibiotic and concentration, and this was outside the scope of this project. Further, in  
318 order to confirm stable transfection, one should investigate integration of the plasmid genes into  
319 the host genome by Southern blot or PCR and sequencing methods. Given that expression of the  
320 YFP gene was detected 5 days post-transfection via RT-PCR (Fig. 5), which represents stability

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321 over at least 5-6 generations, it is possible that this plasmid integrated into the nuclear genome,  
322 but this would need to be confirmed with Southern blotting. Similarly, we established successful  
323 transfection of the choanoflagellate *Monosiga brevicolis* using the microfluidic system with the  
324 same plasmid, pEYFP-Mitotrap, which was expressed for at least 4 days post-transfection (data  
325 not shown). This was supported by RT-PCR but not microscopically, due to overlap between the  
326 strong cell autofluorescence signal and the reporter gene signal.

327 **The square-wave system.** The square wave system with the specific electric field  
328 conditions given in Table 2 successfully delivered pUB-GFP plasmid DNA into the *P. caudatus*  
329 cytoplasm and achieved transient GFP expression with a transfection efficiency of about 40 %  
330 (Fig. 4c and 4d). In Under these conditions, both poring and transfer pulses had an equal electric  
331 voltage strength of 99 V ( $E = 500$  V/cm). In contrast, attempts to establish transgenic *P. caudatus*  
332 using the previously applied electroporation parameters for diatom transformation (Miyahara et  
333 al., 2013) with high poring pulses voltage (150 V or 300 V) and low transfer pulses voltage (8 V)  
334 were unsuccessful. These results suggested that even though *P. caudatus* and the diatom  
335 *Phaeodactylum tricornutum* are single-celled marine eukaryotes, they possess different cell  
336 characteristics and therefore a different electric field strength and pulse number are required for  
337 successful intracellular delivery of exogenous DNA.

338 **The exponential decay system.** The exponential decay electroporation system was also  
339 used successfully to establish transgenic *P. caudatus* using two plasmids: the pEF-GFP and the  
340 pEYFP-Mitotrap (Fig. 3a and 3b). Comparison of results for the square wave and the exponential  
341 decay systems shows the exponential decay system results in a lower transformation efficiency of  
342 5 %, and that the survival rate of the electroporated cells after exponential decay pulses at 800 V  
343 ( $E = 4,000$  V/cm) is less than 50 % (based on light microscopy observation of swimming cells).



344 Relative to the square wave system, these results clearly indicate the increased effectiveness of the  
345 lower voltages and multiple pulses produced by the square wave electroporation system for  
346 delivering the extracellular DNA to larger numbers of cells with minimum cell damage.

347 **The microfluidic electroporation system.** The microfluidic electroporation system  
348 resulted in the highest transfection efficiencies ranging from 20 % to 50 %. The applied electric  
349 fields were much smaller than the ones employed during the SYTOX<sup>®</sup> assay in order to increase  
350 cell viability. We demonstrated successful *P. caudatus* transfection employing electric fields of  
351 1,500 V/cm, resulting in transformation efficiencies of 30-40 %, and 2,250 V/cm, resulting in  
352 transformation efficiencies of 40-50 % efficiency using 5 ms pulse durations in MilliQ water and  
353 the bilaterally constricting channel geometry. Additionally, by decreasing the electric field to  
354 1,000 V/cm and by employing longer 20 ms pulses, we achieved 20-30 % transfection efficiencies  
355 in 50 % cytomix buffer using the straight channel constriction. These results demonstrate that  
356 different geometric constrictions can be used successfully to modulate the electric field that the  
357 cell is exposed to for successful transfection.

358 The major advantage of the microfluidic platform is that it allows continuous flow-through  
359 transfection in comparison to traditional, commercially available cuvette-based technologies,  
360 while achieving comparable or better transfection efficiencies. Additionally, since the flow-  
361 through transfection process is continuous in nature, there is flexibility in the sample volume. This  
362 has exciting implications for processing large sample volumes (> 10 ml) within minutes,  
363 increasing throughput by 100 times in comparison to cuvettes (Garcia et al., 2017). This has  
364 advantages for future genome editing applications including library generation, and the ability to  
365 transfect cells directly from aqueous environments.

366

## 367 **Conclusions**

368       Development of successful transfection protocols for marine protists will enable advances  
369 in our understanding of their ecology. Here we successfully transfected *P. caudatus* for the first  
370 time using three different electroporation-based transfection methods and three different DNA  
371 plasmids pEF-GFP, pUB-GFP, and pEYFP-Mitotrap. Between the two traditional cuvette-based  
372 technologies (exponential decay and square wave), multi-pulse square wave electroporation  
373 resulted in higher transformation efficiency and cell viability. The microfluidic electroporation  
374 system produced the highest transfection efficiency (20-50%) when the optimal combination of  
375 buffer, electric field, and flow rate (among those tested in this study) was employed. This implies  
376 that microfluidic transfection holds great promise for efficiently optimizing and conducting  
377 electroporation of a potentially wide range of microbial eukaryotes. The microfluidic system is  
378 economical and can be installed and easily used by researchers and academics. The device features  
379 hydrodynamically-controlled electric fields that allow cells to experience a time-dependent pulse  
380 waveform that is otherwise difficult to achieve using standard electronics. The ability to efficiently  
381 test a wide range of electroporation parameters, or to quickly transfect a target (or a collection of  
382 targets) with a range of genetic elements has significant advantages over cuvette-based methods  
383 for the field of genome editing. High-throughput transfection technologies such as our  
384 microfluidics system offer the possibility of parallel processing of multiple samples (cultures or  
385 environmental samples), making possible effective investigations into the ecological roles of  
386 protists.

## 387 **Experimental Procedures**

### 388 *P. caudatus* Strain and Growth Media

*Parabodo caudatus* culture (ATCC 50361) was used in this study. This ATCC strain was isolated from a freshwater sediment location, but *Parabodo* is described from marine habitats (Kopylov et al., 1980). Initially, *Parabodo caudatus* was grown in 50 % ATCC seawater media. Subsequently, seawater was replaced with distilled water in order to reduce the high electrical conductivity during the electroporation. Briefly, this is a cerophyl-based media enriched with 3.5 mM sodium phosphate dibasic (Na<sub>2</sub>HPO<sub>4</sub>) and with *Klebsiella pneumoniae* (ATCC-BAA 1706) added as a food source. *K. pneumoniae* is a gram negative, rod-shaped facultative anaerobe bacterium commonly found in animals and the environment, and routinely used as bacterial prey. Cultures were incubated at 22 °C and sub-cultured weekly in fresh T-25 vented tissue culture flasks (Falcon brand, Fisher Scientific) containing 30 ml of fresh media.

#### **Cell Viability Assay After Cytomix Buffer Incubation**

Cell viability of *P. caudatus* in cytomix buffers needed to be tested since they can be cultured in either MilliQ water or ≤ 50 % seawater. Three replicates of *P. caudatus* cultures each twenty-five ml (i.e., biological replicates of *P. caudatus*, defined as different starting culture bottles, although it is noted that all originated from the same starting strain) in logarithmic growth phase ( $1 \times 10^7$  cells to  $1.3 \times 10^7$ ) were harvested by centrifugation at 5000 x g for 30 seconds and re-suspended in 200 µl of 100 % cytomix, 50 % cytomix, or 10 % cytomix. To evaluate survival in these buffers, aliquots of 20 to 30 µl of the cell-buffer mixture were placed on a haemocytometer every 5 minutes for 15 minutes and were imaged under bright field microscopy (Nikon) using a 20x objective. Survival was determined by counting the total number of swimming cells in the haemocytometer and determining the fraction of live cells.

#### **Electroporation Buffer Conductivity**

411 A buffer with low electrical conductivity is recommended to minimize Joule heating during  
412 electroporation. We evaluated the electrical conductivity for the following buffers: 100 % cytomix  
413 (120 mM KCl; 0.15 mM CaCl<sub>2</sub>; 10 mM KH<sub>2</sub>PO<sub>4</sub>; 25 mM HEPES; 2 mM EGTA; 5 mM MgCl<sub>2</sub>;  
414 pH adjusted to 7.6 with KOH), 50 % cytomix (in MilliQ water), 10 % cytomix (in MilliQ water),  
415 10 % seawater, 1 % seawater, or 100% MilliQ water, at four different voltages (300 V, 500 V,  
416 800 V, and 1000 V). Since the exponential decay system uses 2-mm gap cuvettes, the electric  
417 fields result in 1,500 V/cm, 2,500 V/cm, 4,000 V/cm, and 5,000 V/cm, respectively, after  
418 computing the voltage-to-distance ratio. Electric field amplitude and pulse duration were measured  
419 for each electroporation event with parameters given in Table S1. For square wave we followed a  
420 published protocol for diatom transformation by Miyahara *et al.* (Miyahara et al., 2013), which  
421 uses 2-mm cuvettes with applied voltages of 150 V and 300 V. In the microfluidic device we only  
422 tested combinations of *P. caudatus* cells in the presence of SYTOX<sup>®</sup> or DNA as outlined below.

#### 423 **Electroporation Parameters Tested and Post-Electroporation Cell Viability Quantification**

424 Prior to electroporation of *P. caudatus* cells in the MicroPulser Bio-Rad (CN 165-2100)  
425 exponential decay system (Fig. 1), cell pellets from 25 mL of replicate cultures were re-suspended  
426 in 200 µl MilliQ water, 1 % seawater, 10 % seawater, 10 % cytomix, or 50 % cytomix and  
427 transferred to 2-mm gap cuvettes. The cells were electroporated with applied voltages of 300 V  
428 ( $E = 1,500$  V/cm), 500 V ( $E = 2,500$  V/cm), and 800 V ( $E = 4,000$  V/cm). The pulse duration in  
429 milliseconds (ms) after each electroporation was recorded (Table S1). The cells were immediately  
430 transferred to a 1.5 ml Eppendorf tube containing 1 ml of fresh growth media (ATCC 802 medium  
431 prepared with distilled water) for recovery. To determine cell viability, aliquots (20-30 µl) of  
432 electroporated cells were quantified using microscopy for each electric field applied.

433 The NEPA21 transfection system (Bulldog Bio), which utilizes square wave pulses, was  
434 used for electroporation of *P. caudatus* in 2-mm gap cuvettes with identical buffers as used for the  
435 exponential decay experiments. We initially used the same electroporation parameters that were  
436 successfully applied previously for transformation of diatoms (Miyahara et al., 2013). However,  
437 these high applied voltages of 300 V or 150 V were found to compromise *P. caudatus* cell viability  
438 so modifications were necessary with a lower applied voltage (Table 2). It is important to note that  
439 transformation in *P. caudatus* was most successful when we employed ‘poring’ ( $t = 5$  ms) and  
440 ‘transfer’ ( $t = 50$  ms) pulses of the same amplitude (99 V) but with different pulse durations.

441 We recently developed a continuous flow system to transform microorganisms in high  
442 throughput in a microfluidic device (Garcia et al., 2017). This system employs microfluidic  
443 channels that contain a bilateral constriction between the inlet and outlet electrode connections  
444 ( $length = 3.0$  mm,  $width_{min} = 50$   $\mu$ m,  $width_{max} = 2.0$  mm, and  $height = 100$   $\mu$ m). The constriction  
445 amplifies the electric field under an applied voltage between the inlet and outlet electrodes to levels  
446 sufficiently high to induce electroporation. As opposed to the previous two systems that deliver  
447 uniform electric fields in static cuvettes, this system drives cells through the constriction, which is  
448 the region of highest electric field. During *P. caudatus* transfection, the cells were driven through  
449 the microfluidic device at flow rates of 50  $\mu$ L/min and 500  $\mu$ L/min, which correspond to residence  
450 times (i.e., pulse durations) of 20 ms and 2 ms, respectively. Square wave pulses with, for example,  
451 5 ms ON and 5 ms OFF cycles (50 % duty cycle) are applied to the microchannel through the  
452 dispensing needle. Therefore, the cell viability cannot be accurately evaluated since only 50 % of  
453 the cells experience the electric field. The pulses are delivered from electrodes with alternating  
454 polarity between the pulses to reduce electrolytic effects at the electrode-buffer interface (Fig. 1  
455 and Fig. S1). After flowing through the microchannel (See Supplementary Video 2), each 200  $\mu$ L

cell sample is added to a 1.5 ml Eppendorf tube containing 1 ml of fresh growth media for cell recovery. The applied voltages we evaluated had amplitudes of 250 V ( $E_{max} = 1,500$  V/cm), 375 V ( $E_{max} = 2,250$  V/cm), and 500 V ( $E_{max} = 3,000$  V/cm) for each polarity. The non-uniform constriction in the microfluidic devices generates a variable electric field that is capable of transfecting cells while minimizing exposure to the highest electric field.

#### **Electroporation Protocol Optimization with SYTOX® Blue**

We used the SYTOX® Blue dead cell stain (Thermo Fisher Scientific) to initially determine pulse parameters that induce electroporation for *P. caudatus*. The SYTOX® Blue dye cannot penetrate the plasma membrane of living cells, but easily penetrates compromised plasma membranes, such as those induced by electroporation. Thus, the only cells that fluoresce are those that are exposed to an electric field strength and duration within and above the cell-specific critical electroporation threshold. *P. caudatus* cultures at logarithmic growth phase ( $1 \times 10^7$  cells to  $1.3 \times 10^7$ ) were harvested by centrifugation at 5000 g for 30 s. Cells were re-suspended in 200  $\mu$ L of MilliQ water, 1 % seawater, 10 % seawater, 10 % cytomix, or 50 % cytomix and mixed with SYTOX® Blue dead cell stain to a final concentration of 5  $\mu$ M. Cells were incubated for 2 min, then electroporated with exponential decay or microfluidic systems using different electroporation parameters. Two to three biological replicates (i.e., cells mixed with one of the tested buffer) were used for each of the tested applied voltages (technical replicates). In the exponential decay system we applied voltages of 300 V ( $E_{max} = 1,500$  V/cm), 500 V ( $E_{max} = 2,500$  V/cm), and 800 V ( $E_{max} = 4,000$  V/cm). For the microfluidic device we applied voltages of 500 V ( $E_{max} = 3,000$  V/cm) and 1,000 V ( $E_{max} = 6,000$  V/cm) (Fig. 2). The applied voltage and pulse duration were measured for each electroporated sample and are shown in Table 1. For the exponential decay, cell integrity was confirmed using a bright field microscope (Nikon) and 20x

479 objective (Fig. 2b-2d). The bright blue signal was detected using a fluorescence microscope  
480 equipped with DAPI filter set. For the microfluidic system we were able to confirm the conditions  
481 that lead to successful entry of the SYTOX<sup>®</sup> Blue dye in real-time (Fig. 2e and 2f).

## 482 **Plasmid Selection and Preparation**

483 Three plasmids were obtained from Addgene ([www.addgene.org/](http://www.addgene.org/)). pEYFP-Mitotrap  
484 (CMV mammalian and yeast promoter, the Tom70p gene targeting the outer membrane of the  
485 mitochondria in yeast and mammalian cells, and the YFP reporter) was a gift from Margaret  
486 Robinson (Addgene plasmid # 46942; (Robinson et al., 2010)); pEF-GFP (EF1 alpha promoter  
487 from mammalian cells for expression of GFP) and pUB-GFP (mammalian Ubiquitin C promoter  
488 for expression of GFP) were gifts from Connie Cepko (Addgene plasmid # 11154 and # 11155,  
489 respectively; (Matsuda and Cepko, 2004)). These plasmids were used to assess the transcriptional  
490 activity of those promoters and pEYFP-Mitotrap was used to assess whether Tom70p would only  
491 be expressed within the kinetoplast (a dense DNA-containing granule within the cell's single  
492 mitochondrion). Plasmids were purified from 100 mL cultures grown overnight in standard Luria  
493 Bertani liquid medium (Cold Spring Harbor Protocols 2006) with the appropriate selection marker.  
494 Purification was done according to the manufacturer's protocol for the Plasmid Midi Kit (Qiagen,  
495 Germantown, MD), with the following modifications: 1) Each 100 mL culture was split into two  
496 50 mL volumes and centrifuged at 4,500 rpm for 20 min at 4°C to pellet bacterial cells; 2) Each  
497 half went through the lysis steps separately, and the lysate was pooled after neutralization; 3)  
498 Pelleting of precipitated DNA was done by centrifugation at 4,600 rpm for 60 min at 4°C; 4) Each  
499 2 mL volume of pellet (in 70 % ethanol wash) was split into two 1 mL volumes, centrifuged at  
500 15,000 X g for 10 min at 4°C, and the supernatant decanted; and 5) Dried DNA pellets were re-  
501 suspended in 50 µL of nuclease-free water, and the two 50 µL volumes were combined for each

sample. Purified plasmid DNA was quantified using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and stored at -20°C until use. The success of our plasmid preparations was confirmed by PCR prior to use in transfection experiments.

**Transfection of *P. caudatus*:**

*P. caudatus* cells were grown to logarithmic phase ( $1 \times 10^7$  cells to  $1.3 \times 10^7$ ) and harvested by centrifugation at 5000 X g for 30 s, re-suspended in 200 µl cytomix (50 % in distilled water), mixed with 20 to 40 µg of plasmid, and then transferred into an electroporation cuvette (2.0-mm gap) for electroporation with the exponential decay system and the square wave electroporation system. For the microfluidic system, cells in cytomix buffer were aspirated into 1/16 inch tygon tubing (McMaster-Carr) prior to being delivered into the microchannel. We carried out a minimum of ten trials of each combination of electroporation conditions tested using the three platforms; however, only the successful transformation parameters are summarized in Tables 1 and 2. Electroporation parameters that were not successful are included in Table S1 for the exponential decay system and Table S2 for the microfluidic system.

**RT-PCR Confirmation for Expression of Plasmids in *P. caudatus***

Total RNA was isolated from transformed *P. caudatus* cells using the RNEasy Mini Kit (Qiagen, Hilden, Germany). Cells were filtered onto a Durapore® PVDF 0.45 µm-pore size filter (EMD Millipore, Billerica, MA). The filter was placed in 500 µL RLT lysis buffer (RNA Isolation Kit, Qiagen, Hilden, Germany) with 143 mM β-mercaptoethanol and vortexed. Following 10 min incubation at room temperature, 350 µL of 100 % ethanol were added and the lysate was purified using RNEasy Mini Kit according to the manufacturer's instructions.

Purified RNA then underwent two rounds of DNase treatment (Jones et al., 2007). First,



524 the Turbo DNA-free™ Kit (Ambion®, Thermo Scientific, Waltham, MA) was used with the  
525 following modifications: 1) A total of 2 µL DNase was added, 1 µL each time, with each addition  
526 followed by a 30 min incubation at 37 °C; and 2) 0.2 µL volumes of DNase inactivation reagent  
527 were used. Next, the RNase-Free DNase Set was used in combination with the RNEasy Mini Kit  
528 (Qiagen, Hilden, Germany) to perform an on-column DNase digestion, followed by column-based  
529 purification, according to the manufacturer's instructions.

530 First-strand cDNA synthesis and PCR amplification were performed using the OneTaq®  
531 RT-PCR Kit (New England Biolabs, Ipswich, MA). The appropriate reverse primer (0.5 µM final  
532 conc.; See Table S3) and 5 µL RNA were used for reverse transcription. Control reactions were  
533 performed with water in place of the reverse transcriptase enzyme mix. cDNA was amplified in a  
534 25 µL PCR reaction, with final primer concentrations of 0.2 µM. Thermocycling conditions were  
535 as follows: 30 sec at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 68 °C; and a final  
536 extension for 5 min at 68 °C. PCR primers targeting expression of the GFP or YFP reporter gene  
537 (Table S3) were used. PCR products were visualized by gel electrophoresis, with purified plasmid  
538 as a positive control. Amplified PCR products at the expected size of 367-bp for YFP and GFP  
539 genes were documented (Fig. 5).

#### 540 **Supplementary Methods:**

##### 541 **Soft Lithography Protocol for Microfluidic Device Fabrication**

542 Soft lithography is employed in order to fabricate devices with microscale features. This  
543 process creates a master stamp from photomasks that can be used to create devices repeatedly. The  
544 photomasks are designed in AutoCAD 2014 (Autodesk, San Rafael, CA) with bilaterally  
545 converging or straight geometries, and are printed by Fine-Line Imaging, Inc. (Colorado Springs,

546 CO). The microchannels are microfabricated using soft lithography techniques described by  
547 Garcia *et. al* (Whitesides et al., 2001; Garcia et al., 2016). Briefly, SU-8 (SU-8 2050, Micro-Chem,  
548 Westborough, MA) molds are patterned on silicon wafers with standard photolithography.  
549 Afterwards, the surfaces of the SU-8 master mold are treated for 2 hours with tridecafluoro-1,1,2,2-  
550 tetrahydrooctyl-1-trichlorosilane (Sigma Aldrich, St. Louis, MO) under vacuum before being used  
551 for molding. Next, the SU-8 master mold polydimethylsiloxane (PDMS, Sylgard 184, Dow  
552 Corning, Midland, MI) was used at a 10:1 ratio after 2-hour vacuum for removal of air bubbles in  
553 the polymer. The PDMS devices are bonded to a glass substrate after a 45 second plasma treatment  
554 and placed overnight in an oven at 75 °C prior to subsequent experiments.

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656

657 **Figure 1:** Electric field waveforms employed for transient and stable transfection of *Parabodo*  
658 *caudatus*. a) Three independent electroporation systems were used for reproducible transfection,  
659 including our microfluidic electroporation platform (Garcia et al., 2017), the NEPA21 square-  
660 wave transfection system (Bulldog Bio), and the MicroPulser™ exponential decay electroporator  
661 (Bio-Rad). b) The signature waveforms for the NEPA21 square wave transfection system include  
662 both ‘poring’ and ‘transfer’ pulses for electroporation. Note: The time scale in Fig. 1a is a zoomed-  
663 in version of the red-dashed box from Fig. 1b.

664  
665 **Figure 2:** Permeabilization confirmation of *P. caudatus* cells with SYTOX® Blue. a) Phase  
666 contrast and b-d) fluorescence images of *P. caudatus* electroporated with 5 µM SYTOX® Blue  
667 nucleic acid stain at 300 V ( $E_{max} = 1,500$  V/cm), 500 V ( $E_{max} = 2,500$  V/cm), and 800 V  
668 ( $E_{max} = 4,000$  V/cm), respectively, using the exponential decay electroporation system in 2-mm  
669 cuvettes. Fluorescence images e) before and f) after electroporation in the microfluidic system  
670 using a single 5 ms exponential decay pulse at 500 V ( $E_{max} = 3,000$  V/cm).

671  
672 **Figure 3:** Microfluidic transfection of *Parabodo caudatus*. a) *P. caudatus* (brightfield), b) transient  
673 pEYFP-Mitotrap transfection at 250 V ( $E_{max} = 1,500$  V/cm), c) transient pUB-GFP transfection  
674 using 375 V ( $E_{max} = 2,250$  V/cm), d) autofluorescence control for *P. caudatus*, e) transient  
675 transfection using pEF-GFP and 313 V ( $E_{max} = 1,000$  V/cm) in the straight channel, and f) merged  
676 image of brightfield and fluorescence image from e) for visualizing cell morphology.

677



678

679 **Figure 4:** Exponential decay and square wave transfection of *Parabodo caudatus*. Fluorescence  
680 imaging confirmation of *P. caudatus* after a) transient transfection with pEF-GFP using the  
681 MicroPulser™ exponential decay electroporator, after b) transient transfection with pEYFP-  
682 Mitotrap using 800 V ( $E_{max} = 4,000$  V/cm) in the exponential decay electroporator, and after c)  
683 transient transfection with pUB-GFP using the NEPA21 square-wave transfection system at 99 V  
684 ( $E_{max} = 500$  V/cm). Panel d) shows merged fluorescence image from c) with the brightfield image.

685

686 **Figure 5:** Gel electrophoresis image showing the RT-PCR results detecting reporter genes  
687 expression in *P. caudatus* transformants. (L) 1kb ladder (Invitrogen, cat. #10787018); (1) GFP  
688 expression profile in *P. caudatus* cells transformed with pUB-GFP plasmid at 375 V using the  
689 microfluidic electroporation system; (2) Control reaction was performed without addition of RT  
690 to verify the absence of DNA in the RNA preparations in *P. caudatus* cells transformed with pUB-  
691 GFP transient plasmid using 375 V with the microfluidic electroporation system; (3) pEYFP  
692 expression profile in *P. caudatus* cells transformed with pEYFP-Mitotrap plasmid at 250 V with  
693 the microfluidic electroporation system; (4) Control reaction was performed without addition of  
694 RT to verify the absence of DNA in the RNA preparations in *P. caudatus* cells transformed with  
695 withpEYFP-Mitotrap plasmid at 250 V with the microfluidic electroporation system; (5) PCR  
696 negative control; (6) PCR positive control using the GFP plasmid DNA. The PCR products were  
697 separated on a 1% agarose gel, visualized under UV light, and DNA fragments of both reporter  
698 genes were at the expected size of 367-bp.

699

700 **Figure S1:** Representative 5-ms square waveform delivered with alternating polarity in the  
701 microfluidic device at a 95 % duty cycle. This device geometry results in a ~6x amplification of  
702 the applied voltage in the narrowest portion of the constriction. Therefore, the applied voltage of  
703 250 V presented here results in a maximum electric field ( $E_{max}$ ) of 1,500 V/cm in the  
704 microfluidic device.

705

706 **Figure S2:** Graphical representation of the microfluidic device used in this study for continuous  
707 flow-through transfection of *P. caudatus*. The device exhibits inlet (green) and outlet (red) fluidic  
708 ports that also serve as electrodes to generate the electric field within the bilateral constriction.

709

710 **Supplementary Video 1:** Microfluidic electroporation of *Parabodo caudatus* in the presence of  
711 5  $\mu$ M SYTOX<sup>®</sup> Blue nucleic acid stain using an applied voltage of 1,000 V (20X magnification)  
712 demonstrates successful intracellular delivery of dye due to real-time fluorescence detection.

713

714 **Supplementary Video 2:** Microfluidic electroporation of *Parabodo caudatus* with pUB-GFP  
715 driven at 500  $\mu$ L/min with an applied voltage of 375 V ( $E_{max}$  = 2,250 V/cm) and a 50 % duty cycle  
716 resulted in 20-30 % transfection efficiency.

717